NOVEL PEPTIDES AND POLYPEPTIDES USEFUL FOR REGENERATING THE NERVOUS SYSTEM

The present invention relates in particular to novel peptides and polypeptides useful in particular as medicines in therapeutic treatments involving the regeneration of the nervous system cells, for treating neuroblastomas, and also useful as additives in the cultures of nerve cells.

Many proteins comprising repeating units which are called thrombospondin type I units (TSRs) have been identified during the past few years. It can be said that these proteins have highly varied activities depending on the biological system in which they are involved. There may be mentioned, as the best studied and therefore the best known examples, the CS proteins (of circumsporozoite) which allow binding to the hepatic cells of the agent for the propagation of malaria, the plasmodium falciparum sporozoite (WO 94/06646) and the thrombospondin secreted by the blood platelets which are involved in the phenomena of thrombosis and angiogenesis (EP 443 404).

In fact, this thrombospondin type 1 unit (TSR) comprises, in all the proteins studied so far and previously mentioned, about 60 amino acids (AA) some of which, like cysteines (C), tryptophans (W), serines (S), glycines (G), arginines (R) and prolines (P) are highly conserved (see below the alignment of these conserved AAs in a few proteins).

Some synthetic peptides, deduced from these TSR units, have valuable biological properties. Thus, the CSVTCG units allow the adhesion of the plasmodium sporozoites to the hepatic cells, the CSVTCG and WXXW units allow cellular attachment in other biological models, BBXB (B being a basic amino acid) binds heparin and finally WSXWS binds certain growth factors.

F-spondin has been described and its sequence has been aligned with that of thrombospondin in Klar et al., (1992), Cell, 69, 95-110.

The general characteristics of SCO-spondin are described in particular in the article by Monnerie et al. (submitted) and the article by Gobron et al., (1996), Journal of Cell Science, **109**, 1053-1061, 1996. In particular, the alignment of the sequence of SCO-spondin has revealed homologies with proteins such as thrombospondin 1 and 2 (see sequence, alignment page 1057 of Gobron et al., (1996), J. of Cell Science 109, 1053-1061, incorporated into the description by reference).

The novelty of the present invention consists in the identification and the selection of a novel peptide which is active in the regeneration of the nervous system, whose sequence is derived from one of the TSRs of SCO-spondin.

More particularly, the present invention relates to a peptide or polypeptide having the formula:

-W-S-A_1-C-S-A_2-C-G- (SEQ ID No. 1)

in which A_1 and A_2 are amino acid sequences comprising 1 to 5 amino acids, with the exception of the peptides or polypeptides having one of the following sequences

-W-S-P-C-S-V-T-C-G- (SEQ ID No. 2)
-W-S-S-C-S-V-T-C-G- (SEQ ID No. 3)
-W-S-Q-C-S-V-T-C-G- (SEQ ID No. 4)

It should be recalled that in the description as a whole, "amino acid" is understood to mean both the natural amino acids and the non-natural amino acids. "Natural amino acid" is understood to mean the amino acids in the L form which can be found in natural proteins, that is to say alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. However, the present invention also relates to the non-natural amino acids,

that is to say the preceding amino acids in their D form, as well as the homo forms of some amino acids such as arginine, lysine, phenylalanine and serine or the nor forms of leucine or valine.

It is also possible to envisage using other amino acids such as, for example:

Abu : alpha-aminobutyric acid
Agm : agmatine
Aib : alpha-aminoisobutyric acid
F-trp : N-formyl-trp
sarcosine
statine
ornithine
desaminotyrosine.

Desaminotyrosine is incorporated at the N-terminal end whereas agmatine and statin are incorporated at the C-terminal end of these peptides.

Preferably, the peptides according to the present invention A_1 is proline or X_1-W-X_2-X_3 (SEQ ID No. 5) where X_1, X_2, X_3 are chosen, independently of each other, from G, S and C, that is to say small amino acids.

Still preferably, A_1 is X_1-W-S-X_3 (SEQ ID No. 6) and A_2 is chosen from RS, VS and VT.

The reasons for these choices will emerge on reading some examples.

Preferably, the polypeptide according to the present invention has the following structure:

-W-S-X_1-W-S-X_2-C-S-A_2-C-G- (SEQ ID No. 7)

The preferred peptide has the following structure:

-W-S-G-W-S-S-C-S-R-S-C-G- (SEQ ID No. 8)

Preferably, the peptides and polypeptides according to the present invention will have the following structure:

Y-W-S-A_1-C-S-A_2-C-G-Z (SEQ ID No. 9)

in which Y and Z constitute the N- and C-terminal ends of the peptide, or comprise amino acid chains having

less than 6 amino acids, or comprise chains of compounds which are not amino acids.

This corresponds to the peptide per se or to a peptide in which the Z and Y ends enhance the pharmacological activity or ensure a better penetration or bioavailability of the active ingredient; thus, it is possible to envisage in the Y and Z ends the use of hydrophilic components which make it possible, where appropriate, to cross certain biological barriers, or alternatively, on the contrary, to envisage more hydrophilic sequences which will allow a better solubilization of the products involved.

Finally, the modification of the ends can facilitate the incorporation of these products into particular galenic forms such as, for example, liposomes or microparticles.

The present invention also relates to DNA expression vectors characterized in that they are capable of expressing the preceding peptides or polypeptides.

The DNA sequences encoding the preceding peptides or polypeptides can be easily determined from amino acid sequences or based, for example, on the natural sequences as will be described in the present application.

The vectors for administration may consist of naked DNA vectors, plasmid vectors, viral vectors or alternatively synthetic vectors.

These are known technologies which will not be described in detail.

The use of these expression vectors makes it possible to express *in situ* the peptides or polypeptides involved and, in some cases, is likely to enhance their activity.

Constructs will of course be chosen which exhibit, if possible, specificity for the nerve cells, since they are the preferred targets for the polypeptides according to the present invention.

The peptides and polypeptides according to the present invention may be prepared by any appropriate method, in particular they may be obtained by chemical synthesis, but it is also possible to obtain them by the biological route using in particular the vectors mentioned above in appropriate cell cultures.

It should in fact be noted, in this regard, that the polypeptides and peptides according to the present invention may be provided in deglycosylated or glycosylated form if necessary. It should also be noted that in some cases and depending on the method of preparation, it may be necessary to renature some tertiary structures of the peptide.

Finally, the polypeptides according to the present invention can be more particularly used for the manufacture of a medicine with the aim of being administered *in vivo*, in particular in all pathological conditions and traumas requiring regeneration of the nervous system cells, and more particularly of their outgrowths and synapses.

These may be pathological conditions or traumas in which neurodegeneration is observed, but they may also be pathological conditions or traumas in which the regeneration of the central nervous system, in particular of the axons, or of the peripheral nerves is necessary.

Among the neurodegenerative pathological conditions in which the compounds according to the present invention may provide a support, there may be mentioned in particular Alzheimer's disease, multiple sclerosis, Parkinson's disease and the different types of myopathies.

As regards the regeneration of the neuronal outgrowths, in particular of the axons, this may involve in particular accident- or trauma-type problems (section of the spinal cord or of the peripheral nerves).

Likewise, the compounds according to the present invention may be used as additives in certain

cell cultures with the same effects as those mentioned above on the growth of cells.

More particularly, the compounds according to the present invention increase neuritic growth (including the axons) in the cerebral cortex neurons. Inhibition of aggregation and defasciculation of the neurites are noted on the spinal cord neurons and an increase in synaptic contacts is also noted.

"Neuritic growth" is defined as an extension, that is to say growth of the neuron outgrowths, whether the dendritic or axonal outgrowth.

"Aggregation" is defined as a grouping together of the cells forming a cluster.

"Defasciculation" is defined as the result of a decrease in adhesivity between neurites, leading to a loose network of neuronal outgrowths.

"Synaptic contact" is defined as the capacity for a neuronal cell to communicate with another cell, it being possible for the latter to also be neuronal.

In another aspect of the present invention, said peptides or polypeptides may be useful for inducing regression of tumorigenicity during a neuroblastoma.

The nomenclature used to describe the sequence of the present peptide is the international nomenclature using the three-letter code or the one-letter code and where the amino-terminal end is presented on the left and the carboxy-terminal end is presented on the right.

The compositions according to the present invention may be provided in any customary form for pharmaceutical administration, that is to say for example forms for liquid administration in a gel or any other support allowing, for example, controlled release.

Among the compositions which may be used, there should be mentioned in particular the injectable compositions more particularly intended for injections into the meningeal and subarachnoidal spaces.

The most active peptide according to the present invention has the following formula:

Trp-Ser-Gly-Trp-Ser-Ser-Cys-Ser-Arg-Ser-Cys-Gly
(SEQ ID No. 8)

It is soluble in basic aqueous medium, has a molecular weight of 1301 Da and has an amino acid composition of:

			N	N(%)	MW	MW(%)
C	Cys	Cysteine	2	16.7	206	15.8
G	Gly	Glycine	2	16.7	114	8.8
R	Arg	Arginine	1	8.3	156	12.0
S	Ser	Serine	5	41.7	435	33.4
W	Trp	Tryptophan	2	16.7	372	28.6

It was obtained by solid phase chemical synthesis.

However, as was indicated above, it can be obtained by genetic engineering using a host-vector system comprising DNA encoding the peptide taking into account, for example, the degeneracy so as to produce it in a large quantity.

The cDNA sequence encoding the peptide may be presented in the following manner (SEQ ID No. 10):

5′ TGG WSN GGN TGG WSN WSN TGY WSN MGN WSN TGY GGN 3′

A = Adenosine	W = A or T
C = Cytosine	S = G or C
G = Guanosine	Y = C or T
T = Thymidine	M = A or C
N = A, C, G or T	

The peptide thus obtained was identified by microsequencing, HPLC analysis, mass spectrometry and sequencing of the complementary DNA.

It is on this peptide (SEQ ID No. 8) that the experiments described below were carried out.

Example 1 : effect of the peptide SEQ ID No. 8 on the growth of the neurons

Materials and method

Dissociated cell cultures of cerebral hemispheres of 8-day old chicken embryos

The neuronal cultures are obtained from 8-day old chicken embryos. The cerebral hemispheres, after removing the meninges, are cut into small pieces and enzymatically dissociated with 0.25% of trypsin in a PBS saline buffer free of calcium and of magnesium for 15 minutes at 37°C.

The cells are centrifuged at 200 g for 5 minutes in DMEM medium with 20% FCS for the trypsin inactivation. The cells are then filtered on nylon membrane (pore size: 48 microns) and collected in a chemically defined medium free of serum containing a 1/1 mixture of DMEM and Ham's F12 medium supplemented with glutamine (4 mM), glucose (33 mM), penicillin G (50 U/ml), streptomycin sulfate (50 µg/ml) and an N2 supplement of Bottenstein and Sato (1979): putrescine (100 µM), sodium selenite (30 nM), human transferrin (50 µg/ml), progesterone (20 nM), insulin (5 µg/ml) and β-estradiol (1 pM). All the N2 supplements were bought from Sigma.

The cells are plated at a density of 7.5×10^4 cells/cm^2 on 24-well plastic plates. For some experiments, the plastic plates are coated either with fibronectin (24 µg/ml) or with thrombospondin (20 µg/ml). The cultures are incubated at 37°C and under air containing 10% CO_2. The medium is not changed during the experiment. These cultures consist of nearly 95% of neurons.

Cell cultures of spinal neurons

The spinal cords of 6-day old chicken embryos are dissected, freed of their meningeal membrane and cut into small pieces in a phosphate buffer (PBS) free of calcium and of magnesium. After incubation with 0.25% trypsin for 10 minutes at 37°C, the tissue is centrifuged at 200 g for 4 minutes in a growth medium containing 20% fetal calf serum in order to stop the trypsinization. The cells are then dissociated by repeated trituration using a Pasteur pipette and resuspended in a chemically defined medium free of serum as above.

The cells are plated at a density of 7.5×10^4 cells/cm^2 on 24-well plastic culture plates. The cultures are incubated at 37°C and under air containing 10% CO_2. The medium is not changed during the experiments and it has already been shown that this type of cell population contained more than 93% of neurons.

The peptides tested are, in addition to the peptide according to the present invention mentioned above (peptide SEQ ID No. 8), a second peptide according to the invention having the structure:

W-G-P-C-S-V-S-C-G-(SEQ ID No. 11)

then 3 peptides for comparison:

D-C-K-D-G-S-D-E-(SEQ ID No. 12)

R-K-A-R-(SEQ ID No. 13)

and a mixed sequence of the peptide SEQ ID No. 8:

S-S-C-R-S-G-C-W-G-S-S-W-(SEQ ID No. 14).

All these peptides were obtained by synthesis.

Results

In the presence of the peptide SEQ ID No. 8, the neurons aggregate and are essentially connected by bundles of long and thick neurites after 5 days of culture. Furthermore, these cells adhere well to the substrate coated with the peptide with no detachment of the aggregates. By contrast, the control cell cultures, in the absence of the peptide, rapidly detach from the plastic substrate at 5 days of culture. However, on

plastic, only the cortical neurons form aggregates from which very few neurites can be observed, which indicates that the substrate is insufficiently adhesive. The number of neuronal aggregates increases by 9.3% between the control culture and the culture treated with the peptide according to the invention.

Morphometric analysis reveals a significant increase both in the number of neurites per aggregate and in the length of the neurites per aggregate. Moreover, wells of plastic coated with BSA are only very slightly adhesive for the neuronal cells.

The tests carried out with other peptides in comparison with the peptide SEQ ID No. 8 at random give no significant result.

The peptide SEQ ID No. 11 gives lower but, nevertheless, significant results.

Likewise, the tests carried out with the peptide SEQ ID No. 13, which is a consensus sequence for attachment of glycosaminoglycans which is present in a large number of proteins which bind to heparin, as well as the peptides corresponding to type A LDL receptors, gave no representative result.

Moreover, the effect of the peptides according to the present invention SEQ ID No. 8 and No. 11 on cultures at low density was studied. Indeed, it has already been demonstrated that high aggregation could influence neuritic growth in the same manner as the strength of adhesion of the cells to the substrate.

The tests carried out at low density showed that in the absence of aggregation, the two peptides significantly increased the percentage of neuronal cells carrying neurites. In the controls, only 24.4% of the adherent cells had neurites at 4 days of culture whereas 2 and 2.5 times as many appeared in the presence of the peptides SEQ ID No. 8 and No. 11, respectively.

The morphometric analyses revealed a significant increase in each of them both in the number of neurites per cell and the length of the neurites in

the presence of the peptide SEQ ID No. 8 and not the peptide SEQ ID No. 11. Under these conditions, this demonstrates that, even in the absence of neuronal aggregation, the peptide SEQ ID No. 8 and to a lesser degree the peptide SEQ ID No. 11 are capable of promoting the adhesion and the neuritic growth of the cortical neuronal cells.

The effect of the peptide SEQ ID No. 8 of the invention was also studied under various experimental conditions:

In the presence of various substrates, it was possible to demonstrate, for example, that the peptide according to the invention significantly increased the number of neurites per aggregate in well-containing plates coated with thrombospondin and fibronectin, compared with the controls, as well as the length of the neurites per aggregate.

The activity of the peptide SEQ ID No. 8 on the spinal cord cell cultures compared with controls shows that the neurons remain distributed for at least one week *in vitro*. The neurons show prominent neuritic growths forming a network without fasciculation of the neurites. An increase in the number of synaptic contacts between the neurites is observed. By contrast, the neuronal cells of the controls form, in general, small aggregates interconnected by long filaments. The neurites growing from the aggregates form relatively rigid bundles along which essentially simple, bi- or tripolar neurons can be seen.

The other peptides tested under the same conditions show no notable difference compared with the controls.

Example 2 : Effect of the peptide SEQ ID No. 8 on the neuroblastoma derived from NIB104

Materials and method

The cells derived from the NIB104 neuroblastoma were cultured in 24-well plastic plates previously

coated with a film of poly-L-lysine, under conditions similar to those for the primary cultures.

Results

In the presence of the peptide SEQ ID No. 8 according to the present invention, the NIB104 neuroblastoma cells are considerably less numerous than in the control cultures. The appearance of the cells is considerably modified because they acquire a characteristic neuronal phenotype. Morphometric analysis reveals that in the presence of increasing concentrations of peptide in the culture medium, the neuritic growth gradually increases. This response is therefore dose-dependant and indicative of a specific physiological effect.